Effect of ABC transporters on HIV-1 infection: inhibition of virus production by the *MDR*1 transporter

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ABSTRACT **The *MDR*1 multidrug transporter P-gp (P-glycoprotein) is an efflux pump that extrudes diverse hydrophobic drugs and peptides from cells. Since the entry of HIV-1 into cells involves an initial interaction of the viral gp41 hydrophobic peptide with the plasma membrane, a potential effect of P-gp on HIV-1 infectivity was explored. Virus production was greatly decreased when P-gp was overexpressed at the surface of a continuous $CD4^+$ human T-leukemic cell line (12D7) infected with $HIV\text{-}1_{NL4-3}$, a T-tropic molecular clone of HIV-1. P-gp overexpression did not significantly alter the surface expression or distribution of either the HIV-1 receptor CD4 or the coreceptor CXCR4. Reduction of HIV-1 infectivity in P-gp-expressing cells occurred both during the fusion of viral and plasma membranes and at subsequent step(s) in the HIV-1 life cycle.—Lee, C. G. L., Ramachandra, M., Jeang, K.-T., Martin, M. A., Pastan, I., Gottesman, M. M. Effect of ABC transporters on HIV-1 infection: inhibition of virus production by the *MDR*1 transporter. *FASEB J.* 14, 516–522 (2000)**

Key Words: ABC transporters · acquired immunodeficiency syndrome · AIDS · P-glycoprotein · virus entry

Plasma membranes of cells are highly selective permeability barriers containing many proteins that serve as molecular pumps and/or gates. Some of these proteins likely interact with viruses to modulate their entry into cells. The human *MDR*1 multidrug transporter is a 170 kDa plasma membrane glycoprotein (P-glycoprotein or P-gp) capable of binding and energy-dependent extrusion of structurally diverse compounds and drugs, e.g., Vinca alkaloids, anthracyclines, epipodophyllotoxins (reviewed in (1). It comprises 12 transmembrane segments and 2 nucleotide binding domains, and belongs to the ATP binding cassette (ABC) family of transporters. Some members of the family of ABC transporters are primarily involved in peptide transport. These include the *Salmonella typhimurium* oligopeptide permease (2–5), the yeast STE6 protein involved in the secretion of the **a** factor mating pheromone peptide (6), and the mammalian MHC-I antigen-presenting TAP1/2 complex involved in import of peptides into the lumen of the endoplasmic reticulum (7). P-gp is also capable of transporting various hydrophobic peptides, such as the toxic peptide ALLN (8) and various biologically active hydrophobic peptide derivatives, including ionophores, chemoattractants, immunosuppressants (9, 10), and synthetic peptides (11).

Entry of the human immunodeficiency virus type 1 (HIV-1) into host cells is mediated through the viral envelope protein. Initially synthesized as precursor gp160, the HIV-1 envelope protein is proteolytically processed intracellularly into surface gp120 and noncovalently associated gp41 transmembrane proteins. Binding of virion-associated gp120 to CD4 and a coreceptor (e.g., CXCR4 for T-tropic viruses and CCR5 for M-tropic viruses) induces gp41 to undergo conformational changes and results in the fusion of the virus with the target cell (see refs 12). Since entry of HIV-1 into cells involves interaction of the hydrophobic amino-terminal fusion domain of gp41 with the cellular plasma membrane and since P-gp is capable of extruding hydrophobic peptides, including possibly the hydrophobic protein domains essential for viral fusion, we investigated whether the overexpression of P-gp interfered with HIV-1 infectivity. Here we show that P-gp expression in T cells indeed inhibited HIV-1 infectivity both during fusion

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with the plasma membrane and at a later step in the virus life cycle.

MATERIALS AND METHODS

Constructs

Retroviral vectors driven by Harvey murine sarcoma virus LTR carry either the *MDR*1/NEO genes alone or *MDR*1wt/*MDR*1mt genes in a bicistronic format with dihydrofolate reductase (DHFR). The DHFR gene product is translated using the encephalomyocarditis internal ribosomal entry site (IRES). *MDR*1mt contains the mutant residue Asn in place of Asp at position 555 of the molecule (D555N). This mutation eliminates P-gp ATPase activity and results in an inactive pump that can still bind drugs. Introduction of various constructs into 12D7 cells was as described previously (13, 14). 12D7 cells expressing *MDR*1 alone were initially selected at 5 ng/ml vincristine (vinc) and maintained at 10 ng/ml vinc. 12D7 cells expressing NEO were selected in 0.9 mg/ml G418. Those expressing the bicistronic constructs were initially selected in 25 ng/ml methotrexate (MTX) and maintained in 40 ng/ml MTX in media where fetal bovine serum (FBS) was substituted with dialyzed FBS. Once a stable population of cells had been obtained, expression of the transgene (e.g., *MDR*1) remained constant for approximately a month even in the absence of continued drug selection. Hence, the cells were removed from drug selection 1–2 wk before HIV-1 infection experiments, and P-gp expression levels were examined prior to the infection experiments. P-gp expression did not influence growth rates of the transfected cells.

Kinetics of HIV-1 production in 12D7 cells expressing the various genes

Parallel cultures of 2×10^6 12D7 cells separately expressing *NEO*, *MDR*1, *MDR*1wt-DHFR, or *MDR*1mt-DHFR were infected with HIV-1_{NL4-3} at a multiplicity of infection (MOI) of 0.00375. Media containing no cells served as the negative control while the parental 12D7 cells served as a positive control. Virus production was assayed by measuring reverse transcriptase (RT) activity (15) in the supernatants at time intervals as indicated.

Fluorescence-activated cell sorting (FACS) analyses of 12D7 or 12D7-*MDR*1 cells triply stained with antibodies (Ab) against P-gp (MRK16), CD4, and CXCR4 (12G5)

Approximately 2×10^5 cells were incubated on ice for 40 min with 4 μl of either 12G5 Ab or its isotype control. After three washes in Hank's medium containing 1% bovine serum albumin (HB), the cells were incubated with 1 μl of FITC anti-mouse IgG_{2a} (PharMingen, San Diego, Calif.) for another 40 min. After another three washes in HB, these cells were incubated with either biotinylated MRK16-Fab or its isotype control at 4°C for 40 min. The cells were washed three more times in HB and incubated with 4 μl streptavidin-RPE (Life Technologies, Inc.-Gibco, Gaithersburg, Md.) and 5 μl CD4-APC (Becton Dickinson, Franklin Lakes, N.J.) or its isotype controls. After a final three washes in HB, the cells were resuspended in phosphate-buffered saline (PBS) containing 3.7% formaldehyde for FACS analyses using a FACSort flow cytometer equipped with CellQuest software (Becton Dickinson, San Jose, Calif.). Single antibody-stained and isotype control-stained cells were used as controls.

Cell fusion between *MDR*1-expressing and HIV-1 envelope-expressing cells

A recombinant vaccinia virus (vv) -based transient expression system as described previously (16, 17) was used to study cell fusion. Briefly, effector HeLa cells were coinfected with 10 pfu/cell of VCB21 (vv expressing β-galactosidase under the control of the T7 promoter) and either VCB60 (HIV-1 wild-type envelope expressing vv) or VCB16 (vv expressing mutant HIV-1 envelope, which was deleted in the gp120/gp41 cleavage site rendering the protein nonfusogenic). Target HeLa cells were coinfected with VCB3 (CD4-expressing vv), VTF7–3 (vv expressing T7 RNA polymerase), and either of the following vv: WR (wild-type control), *MDR*1, D555N (amino-terminal ATP binding site mutant of *MDR*1), V185 (substrate mutant of *MDR*1 that changes substrate specificity of *MDR*1) (18), or CFTR (19). Infection was initiated in media containing 2.5% FBS at 32°C with gentle shaking every 15 min. After 1.5 h, the cells were trypsinized, washed and resuspended in media containing 10% FBS, and incubated at 32°C overnight. The next day, 1×10^5 effector and target cells were allowed to fuse at 37°C for 2.5 h. Cells were then lysed in 0.5% Nonidet P-40, and aliquots of the samples were mixed with an equal volume of 2× β-gal substrate (chlorphenol red-β-D-galactopyranoside) (CPRG). Rates of substrate hydrolysis (OD/min) were monitored at room temperature by measuring absorbance at 570 nm using a SpectraMax 250 microplate reader (Molecular Devices, Sunnyvale, Calif.). CPRG hydrolysis from mixing of cells containing mutant HIV-1 envelope lacking the gp120/gp41 cleavage site that represents background β-gal activity was subtracted.

Transfection of pNL4–3 DNA into *MDR*1-expressing cells

pHa*MDR*1 plasmid was transfected into KB-3–1, a subclone of HeLa, and selected at increasing concentrations of vincristine (V) (expressed as ng/ml) to obtain escalated expression of P-gp. HeLa cells were cotransfected with pCMV-β-gal, HIV-1_{pNL4-3} DNA, and the various constructs shown in **Fig. 1*A***. Western blotting using anti-P-gp monoclonal Ab, C219, an anti-p24 Ab (from NIAID AIDS Reagent and Reference Program, Bethesda, Md.), or an HIV-1 patient's serum was performed as described (20) to evaluate the expression of P-gp or HIV-1 proteins, respectively. The *MDR*1-reversing agents quinidine (20 μM) and PSC 833 (1 μM) were added to either parental KB-3–1 cells or *MDR*1-expressing KB-V1 before HIV-1_{pNL4-3} DNA was introduced into the cells by transfection. HIV-1_{pNL4-3} DNA was then introduced into these cells by calcium phosphate precipitation (21). Two days later, RT activity (15, 22) was determined in the media, which had been centrifuged to remove floating cells/cell debris. Quantitation of RT activity was performed using the STORM 860 phosphorImaging system. Transfection differences were normalized by cotransfection of pCMV-β-gal plasmid and determination of β-gal activity as described earlier.

RESULTS

P-gp overexpression inhibits HIV-1 production

The effect of P-gp on HIV-1 infectivity in T cells was studied by the stable introduction of various *MDR*1-expressing constructs (13, 14) and controls (Fig. 1*A*) into 12D7, a human CEM T cell line (23), followed

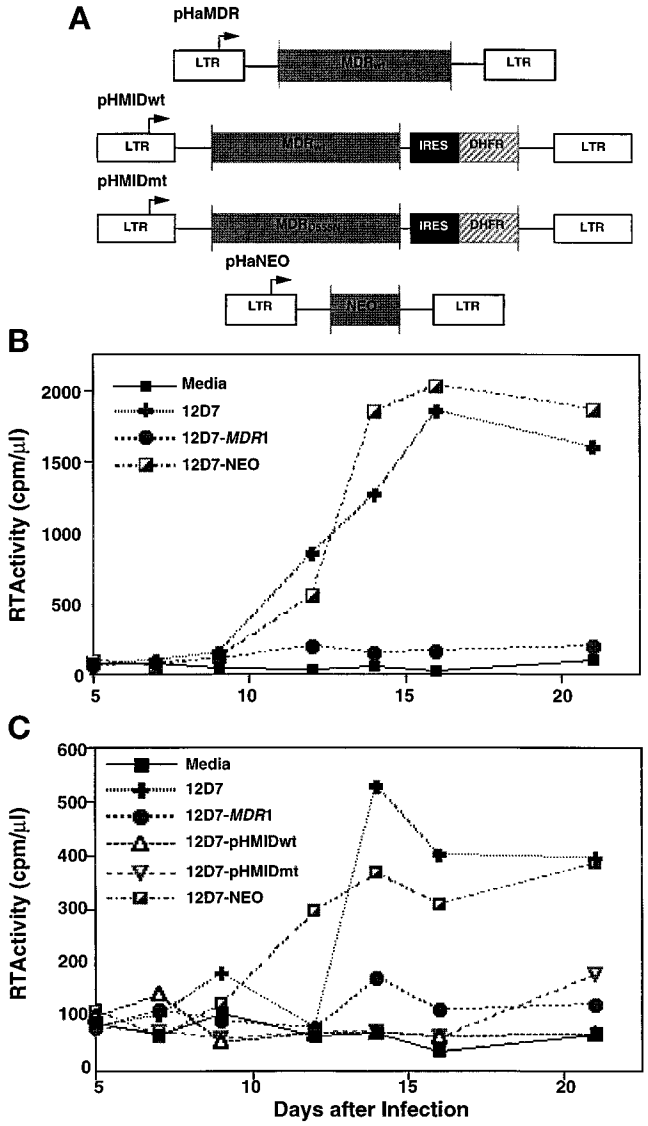


Figure 1. Infection of variously transduced 12D7 T cells (11) by HIV-1_{NL4-3}. *A)* Constructs used to obtain stable 12D7 transfectants. *B, C)* Kinetics of HIV-1 production in 12D7 cells expressing the various genes. Parallel cultures of cells stably expressing the indicated genes were infected with 1500 reverse transcriptase (RT) units (expressed as ^{32}P cpm) of HIV-1_{NL4-3} molecular clone per μl (MOI: −0.00375). Parental 12D7 cells served as positive control whereas media blank served as negative control. Virus production was assayed by measuring RT activity in the supernatants at the time intervals indicated. The two graphs represent data from two different experiments. Similar results were seen in five independent experiments.

by infection with HIV-1_{NL4-3}. Figure 1*B* shows that whereas the parental 12D7 and control neomycin (NEO) -expressing 12D7 cells supported virus replication as monitored by production of supernatant RT activity, *MDR*1-expressing 12D7 cells did not. Since extrusion of drugs and peptides by P-gp requires active transport, we evaluated whether functional *MDR*1 is required for this effect. Bicistronic constructs expressing either wild-type (wt) *MDR*1 or *MDR*1 mutated (D555N) at the ATP utilization site (mt) to inactivate P-gp pump function and DHFR were stably introduced into 12D7 cells by selection with methotrexate (24, 25). When these selected cells were infected with the HIV-1_{NL4-3}, neither wt nor mt *MDR*1-expressing cells supported HIV-1 production (Fig. 1*C*). These results suggest that the physical presence of P-gp protein, albeit not functioning as a molecular pump, might be sufficient to block HIV-1 infectivity. Nonetheless, there is a slight possibility that the effects observed with the *MDR*1 mutant in the bicistronic format may be due to the DHFR gene.

The above observation that overexpression of *MDR*1 inhibits HIV-1 entry was further confirmed using a single cycle infectivity assay. HeLa cells, stably expressing CD4 ($CD4^+$-Hela), were transfected with pHa*MDR*1 and selected with vincristine. $CD4^+$-HeLa cells that stably express *MDR*1 were then infected with HIV-1_{NL4-3}, and virus-specific p24 protein synthesis in the infected cells was examined by Western analyses. HIV-1 p24 expression was reduced approximately fivefold in MAGI cells expressing *MDR*1 (data not shown). This result is in agreement with the *MDR*1-induced inhibition of HIV-1 progeny particle production shown in Figs. 1*B, C*.

Since an active P-gp pump does not seem to be required for anti-HIV-1 effects, we asked whether overexpression of *MDR*1 on the cell surface alters the amount and/or distribution of the HIV-1 receptor CD4 and/or the coreceptor CXCR4. No major differences were observed between parental and *MDR*1-expressing 12D7 cells either by three-color FACS analyses (**Fig. 2**) or fluorescence microscopy (data not shown) although three-color FACS did show a very small population of cells that expressed *MDR*1 but exhibited reduced CD4 and CXCR4 surface expression (Fig. 2. 12D7-MDR1 lower leftmost and rightmost). It is unlikely that this subpopulation of *MDR*1-expressing, low CD4/CXCR4-expressing cells was responsible for the significant attenuation of HIV-1 infectivity in these cells.

*MDR*1 interferes with HIV-1-induced fusion

Fusion between *MDR*1, CD4-expressing cells and HIV-1 envelope-expressing cells was examined using a previously described, recombinant vaccinia virus-based transient expression system (16, 17). Effector HeLa cells were coinfected with vaccinia viruses expressing HIV-1 envelope and a β-galactosidase (β-gal) reporter gene under the control of a T7 promoter. Target HeLa cells were coinfected with vaccinia viruses that express CD4 receptors, *MDR*1 or its mutants/family members and T7 RNA polymerase. Fusion between the effector and target cells results in activation of the β-gal reporter gene, which is monitored by the hydrolysis of chlorphenol red-β-D-galactopyranoside (CPRG) (16).

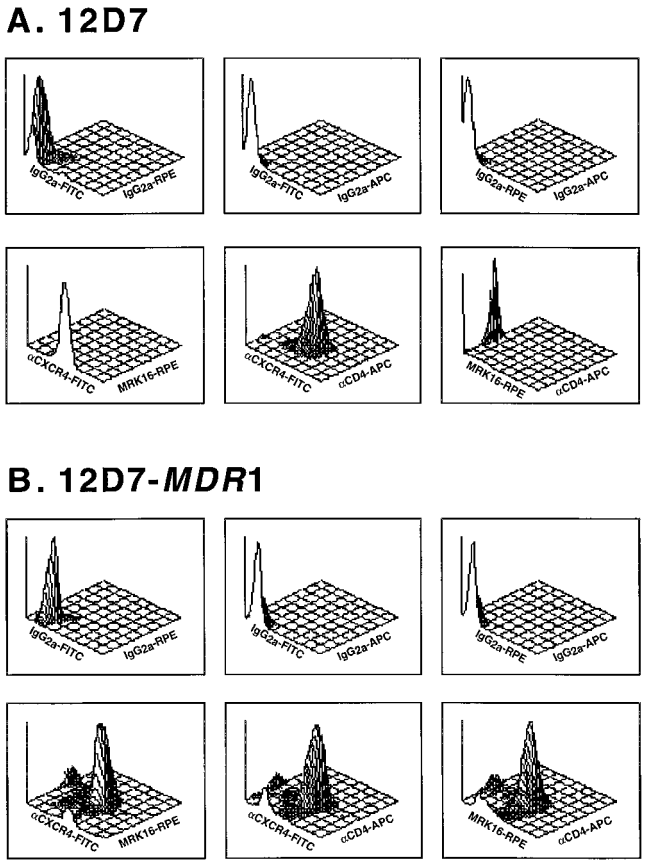


Figure 2. FACS analyses of 12D7 or 12D7-MDR1 cells triply stained with antibodies (Ab) against P-gp (MRK16), CD4, and CXCR4 (12G5). *A)* Data from 12D7 cells stained with the respective antibodies. *B)* Data from 12D7-*MDR*1 cells stained with the respective antibodies. The top three panels of *A* and *B* represent cells stained with control antibodies alone and the bottom three panels in *A* and *B* represent cells stained with respective antibodies. X and Y axes in each panel show the fluorescence intensity from the respective antibodies that have been labeled with the respective fluorescent probes as indicated. The *z* axis shows the number of cells expressing the indicated level fluorescence. FITC: fluorescein isothiocyanates; R-PE: R-phycoerythrin; APC: allophycocyanin; IgG2a: control antibody; αCD4: CD4 antibody; αCXCR4: CXCR4 antibody; MRK16: *MDR*1 antibody.

Figure 3 shows that fusion was greatly reduced in target cells expressing either wt *MDR*1, *MDR*1 with an inactivating amino-terminal ATP-utilization site (D555N) mutation that renders the transporter inactive or *MDR*1 with a single mutation in the substrate binding site (G185V) (18). These results correlated with the observation that 12D7 cells expressing *MDR*1, *MDR*1-IRES-DHFR, or *MDR*1mt-IRES-DHFR inhibited HIV-1 infection (Fig. 1*B, C*). Surface expression, as monitored by FACS analyses, of P-gp or its mutants, CD4, and CXCR4, was similar in all these cells (data not shown). Cells infected with vaccinia viruses expressing CFTR (19), another member of the ABC family of transporters, also exhibited reduced fusion activity (Fig. 3). Since the amount of CFTR on the surface of cells cannot be compared directly with the amount of *MDR*1 on the surface of cells, it is not possible to compare the extent of inhibition of fusion by these two molecules.

*MDR*1 also interferes with HIV-1 infection at steps downstream of fusion

We also examined whether overexpression of *MDR*1 interfered with steps in the virus life cycle downstream of HIV-1 fusion. To investigate this, the fusion step was bypassed by directly transfecting HIV_{pNL4-3} cDNA into KB-3–1 cells, a subclone of HeLa, or KB-3–1 cells that had been stably transfected with pHa*MDR*1 and selected with various concentrations of vincristine to achieve different levels of P-gp expression. Virus production was determined by measuring reverse transcriptase activity in the media of these cells. Reduction in HIV-1 virus production correlates with increasing expression of P-gp (**Fig. 4*A***). To rule out the possibility that overexpression of *MDR*1 interfered with the synthesis of HIV-1 proteins, parental KB-3–1 or its multidrug-resistant derivative, KB-V1 (expressing high levels of P-gp) were transfected with the $HIV\text{-}1_{pNL4-3}$ molecular clone. Viral proteins inside the transfected cells were determined using Western blot analyses whereas virus production was examined by measuring reverse transcriptase activity in the media of these cells. The major HIV-1 protein bands were detected in KB-3–1

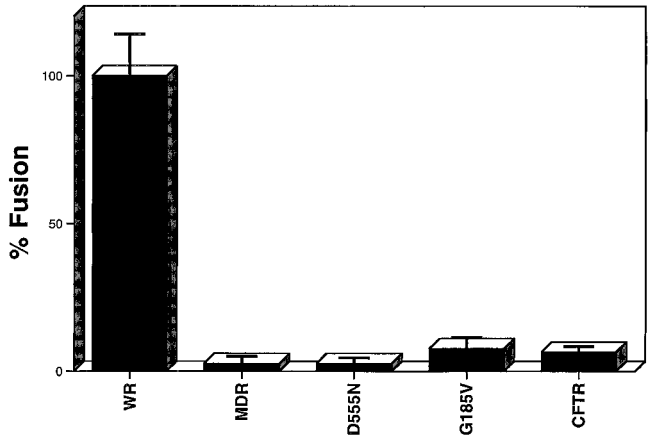
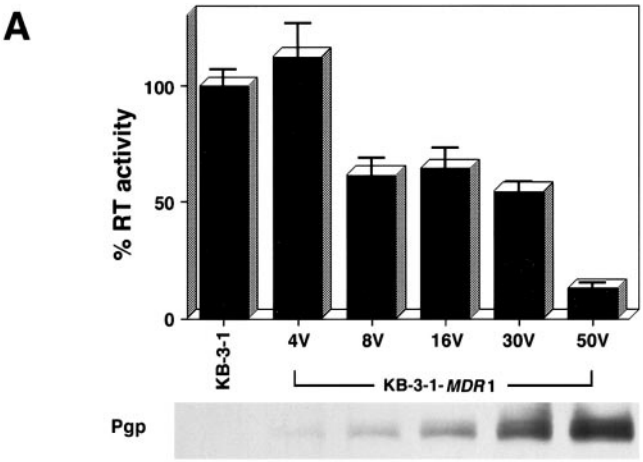
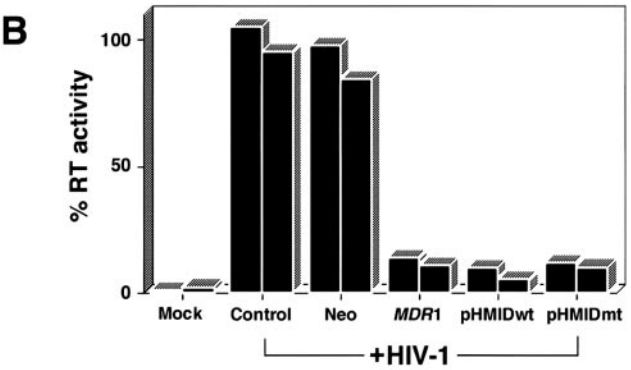


Figure 3. Cell fusion between MDR1-expressing and HIV-1 envelope-expressing cells. Cell fusion was examined using the recombinant, vaccinia virus-based transient expression system as described in Materials and Methods. WR: wild-type control; *MDR*1: wild-type *MDR*1; D555N: amino-terminal ATP binding site mutant of *MDR*1; G185V; substrate mutant of *MDR*1 that changes the substrate specificity; CFTR: cystic fibrosis transmembrane regulator. Results are shown as percent fusion, which represents the mean and standard deviation of the normalized β-gal activity from three samples, expressed as a percentage with respect to effector cells infected with WR virus. Similar observations were made in at least three independent experiments.





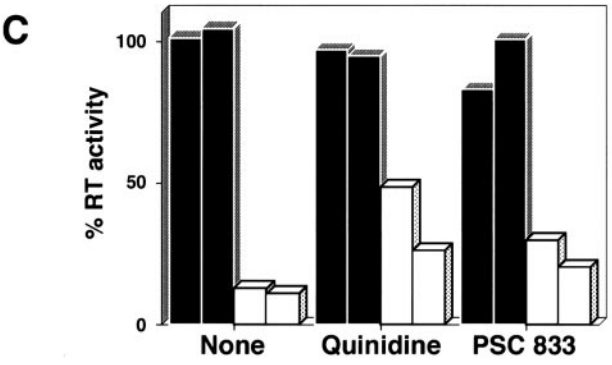


Figure 4. Analyses of steps downstream of fusion via the transfection of pNL4–3 DNA into *MDR*1-expressing cells. *A)* Decrease in HIV-1 production on transfection of pNL4–3 cDNA correlates with increased expression of P-gp. pHa-*MDR*1 plasmid was transfected into KB-3–1, a subclone of HeLa, and selected at increasing concentrations of vincristine (V) (expressed as ng/ml) to obtain escalated expression of P-gp as shown in the lower panel, which represents Western blotting using anti-P-gp monoclonal Ab, C219. pNL4–3 DNA was then introduced into these cells and normalized RT was determined 2 days later. The results represent the mean and standard deviation from four independent transfections expressed as % RT activity, which is the normalized RT activity compared to the mean RT in parental KB-3–1 cells (100% RT is ~950,000 counts). *B)* Functional *MDR*1 is not necessary for the decrease in HIV-1 production from transfected pNL4–3 DNA. HeLa cells were cotransfected with pCMV-β-gal, HIV-1 pNL4–3 DNA, and the various constructs shown in Fig. 1*A*. Normalized RT activity was determined. The results are expressed as % RT activity, which is the normalized RT activity expressed as a percentage of the mean RT activity in parental HeLa cells transfected with HIV-1 (+ salmon sperm DNA) alone. The two bars from each point represent data from duplicate transfections. *C) MDR*1-reversing agents partially reverse the decrease in HIV-1 production in *MDR*1-expressing cells. The *MDR*1-reversing agents quinidine (20 μM) and PSC 833 (1 μM) were added to either parental KB-3–1 cells (dark bars) or *MDR*1-expressing KB-V1 (light bars) before HIV-1_{pNL4-3} DNA was introduced into the cells by transfection. RT activity was determined as described previously (22). Results from duplicate transfections (represented by the two same colored bars) are expressed as % RT activity, which is the normalized RT activity compared to the mean RT activity in the untreated parental KB-3–1 cells.

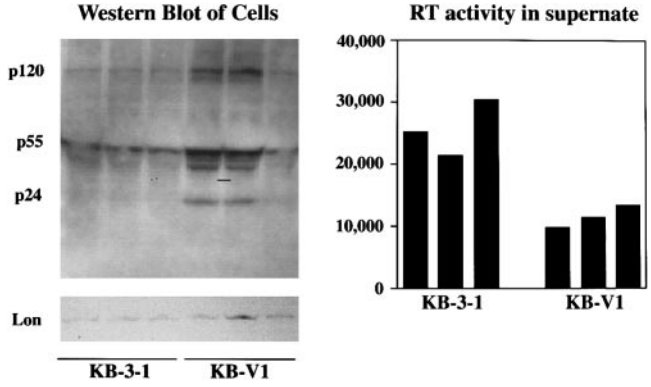


Figure 5. Overexpression of MDR1 does not interfere with the synthesis of HIV-1 proteins. Parental KB-3–1 or its multidrug resistant derivative KB-V1 was transfected with HIV-1_{pNL4-3} DNA. Viral proteins inside cells were determined by Western blot analyses using HIV-1 patient serum. Virus production was determined by assaying RT activity in the media of these cells. The left panel shows Western blot analyses of cells and the right panel shows RT activity in the supernatant of these cells. At the bottom of the left panel is the Western blot from the top panel, which has been reprobed with a control Lon protease antibody to evaluate whether equal amounts of proteins have been loaded into each lane. The left three lanes in each panel represent three parallel transfections of HIV-1_{pNL4-3} DNA into KB-3–1 cells whereas the right three lanes in each panel represent three parallel transfections of HIV-1_{pL4-3} DNA into KB-V1 cells. Similar results were seen in two other independent experiments.

and KB-V1 cells, although there was a reduction of RT activity in KB-V1 cells (**Fig. 5**).

To determine whether functional *MDR*1 is required for this effect, HeLa cells were cotransfected with pNL4–3 cDNA, control pCMV-β-gal, and each of the constructs shown in Fig. 1*A*, and progeny virion production was determined. Although high levels of virus were released after cotransfection of HIV-1 (plus salmon sperm DNA) or HIV-1 and pHaNEO, cotransfection of HIV-1 with either pHa-*MDR*1, pHMIDwt, or pHMIDmt greatly reduced virus production (Fig. 4*B*).

*MDR*1 function requires substrate binding and substrate-induced ATPase activity that results in sub-

strate translocation (pumping). The data presented above indicate that pump function is not needed for the *MDR*1 effect on HIV-1 production. To determine whether *MDR*1-reversing agents, which interfere with substrate binding, can restore HIV-1 infectivity in *MDR*1-expressing cells, KB-3–1 (parental) or its multidrug-resistant derivative KB-V1 were incubated with P-gp inhibitors, quinidine, and PSC 833 and then transfected with pNL4–3 cDNA as described (22). Whereas KB-V1 cells showed reduced virus production compared to parental KB-3–1 cells, incubation of KB-V1 cells with either quinidine or PSC 833 increased virus production, but these agents could not fully restore susceptibility to HIV-1 (Fig. 4*C*). These observations suggest that although a functional *MDR*1 pump may not be required for inhibition of virus production, binding of *MDR*1-reversing agents to the hydrophobic substrate binding site(s) of P-gp interferes in part with the ability of P-gp to block HIV-1 infection.

DISCUSSION

In the present study, we found that the overexpression of *MDR*1 in cells reduces the susceptibility of $CD4^+$ human cells to HIV-1, probably by affecting viral fusion as well as downstream events. This represents the first observation that the expression of a multimembrane-spanning protein inhibits HIV-1 infection. Although we do not yet fully understand the mechanism of inhibition of HIV-1 by *MDR*1, our results indicate that this block cannot be explained by a major down-regulation or gross rearrangement of the HIV-1 receptor CD4 or the coreceptor CXCR4 on the surface of *MDR*1-expressing cells. Furthermore, ATP hydrolysis by P-gp resulting in an active pump is not required for interference with the infection process, since an ATP binding site mutant of *MDR*1 also exhibited similar effects. However, the effect of inhibitors of substrate binding suggests that an active hydrophobic binding domain in P-gp may be at least partially responsible for this phenotype. Our observations could explain a recent report (26) that hematopoietic stem cells are resistant to HIV-1 infection although they express the HIV-1 receptor CD4 and coreceptor CXCR4, since these cells are known to express P-gp (27). These data are also consistent with another recent observation indicating that P-gp overexpressing cells are resistant to other enveloped viruses that enter via the plasma membrane (Y. Raviv, A. Puri, and R. Blumenthal, unpublished results).

Using the recombinant vaccinia virus-based transient expression system for assessing fusion, we have some indications that in addition to *MDR*1, overexpression of other members of the ABC family of transporters—e.g., CFTR (Fig. 4), STE6, or PDR5 (data not shown)—also inhibit HIV-1 envelope fusion. This phenotype could be the result of overexpression of any multimembrane domain-spanning protein or may reflect the ability of each of these ABC family members to form hydrophobic binding sites within the plasma membrane. However, though more extensive studies are needed to evaluate the significance of these observations, the idea that ABC family members might dominantly inhibit HIV-1 infection raises the consideration that some multiply exposed/uninfected individuals might be explained by a gain in P-gp-like function rather than a loss of coreceptor function.

One important implication of these results is that there may be pockets of cells within humans (e.g., hematopoietic stem cells) (26) expressing high levels of P-gp that are resistant to HIV-1 infection. Paradoxically, high P-gp expression also reduces uptake of HIV-1 protease inhibitors (22, 28), so although P-gp-expressing cells may be relatively resistant to HIV-1 infection, once these cells are infected it may be more difficult to eradicate the virus. To produce virus-resistant cell populations, a gene therapy approach could be used to introduce the *MDR*1 gene into these cells. The ability of P-gp to confer multidrug resistance provides a powerful selection both *ex vivo* and *in vivo* for cells transduced with the *MDR*1 gene (1). FJ

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